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Entrapment of lipid vesicles and membrane protein-lipid vesicles in gel bead pores

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Phospholipid vesicles were entrapped in gel beads of Sepharose 6B and Sephacryl S-1000 during vesicle preparation by dialysis. Egg-yolk phospholipids solubilized with cholate or octyl glucoside were dialysed together with gel beads for 2–5 days in a flat dialysis bag. Some vesicles were formed in gel bead pores and vesicles of sufficient size became trapped. Red cell membrane protein-phospholipid vesicles could be immobilized in the same way. Non-trapped vesicles were carefully removed by chromatographic procedures and by centrifugation. The amount of entrapped vesicles increased with the initial lipid concentration and was dependent on the relative sizes of vesicles and gel pores. The largest amount of trapped vesicles, corresponding to 9.5 μmol of phospholipids per ml gel, was achieved when Sepharose 6B gel beads were dialysed with cholate-solubilized lipids at a concentration of 50 mM. In this case the vesicles had an average diameter of 60 nm and an internal volume of 15 μl/ml gel. The amount of vesicles trapped in Sephacryl S-1000 gel beads upon dialysis under the same conditions was smaller: 2.2 μmol of phospholipids per ml gel. Probably most of the gel pores were too large to trap such vesicles. Larger vesicles, with an average diameter of 230 nm, were entrapped in the Sephacryl S-1000 matrix in an amount corresponding to 3.0 μmol phospholipids per ml gel upon dialysis of the gel beads and octyl glucoside-solubilized lipids at a concentration of 20 mM. The internal volume of these vesicles was 22 μl/ml gel. The yield of immobilized phospholipids was up to 19%. The entrapped vesicles were somewhat unstable: 9% of the phospholipids were released during 9 days of storage at 4°C. By the dialysis entrapment method vesicles can be immobilized in the gel beads without using hydrophobic ligands or covalent coupling.

Introduction

Phospholipid vesicles and protein-phospholipid vesicles can be immobilized on hydrophobic derivatives of Sepharose and Sephacryl gel beads [1,2]. A new application has been described recently. After incorporation of a transport protein into the lipid bilayers a chromatographic effect of the transport can be observed ('transport retention chromatography') [2]. In addition, interactions between, for instance, macromolecules and the lipid vesicles can be studied, although non-specific binding to free hydrophobic ligands in the gel beads may be disturbing. The purpose of the present work was to show that vesicles could be mechanically entrapped into gel beads without the use of ligands or covalent coupling. Vesicles of sufficient size became entrapped in gel pores in which they were formed upon dialysis of mixtures of detergent-solubilized lipids and gel beads. Addition of amphiphilic membrane proteins to the dialysis mixture led to the formation of protein-phospholipid vesicles, some of which became trapped in the gel pores. We have determined the amounts and sizes of the immobilized vesicles. Amphiphiles incorporated into the lipid bilayers of the entrapped vesicles have proved to be accessible for binding to proteins (to be reported elsewhere).

Materials and Methods

Materials

Calcein, dithioerythritol, *n*-octyl β-D-glucopyranoside (octyl glucoside) and Tris (Trizma base) were bought from Sigma (St. Louis, MO, U.S.A.). D-[U-^{14}C]Glucose (10 GBq/mmol) and [*carboxyl*-^{14}C]cholic acid (2 GBq/mmol) were purchased from Amersham International (Amersham, U.K.) or New England Nuclear, Du Pont, NEN Products (Boston, MA, U.S.A.). 2-Mercaptoethanol was from Fluka (Buchs, Switzerland).

Abbreviation: DTE, dithioerythritol.

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Other chemicals were as described in Ref 2 Human red cell concentrate was obtained from the Blood Bank at the University Hospital (Uppsala, Sweden) The dialysis tubings were of the regenerated cellulose type, molecular weight cut-off given as M_r 12000–14000, from Viskasi (Chicago, IL, U S A), Sepharose® 6B and Sephacryl® S-1000, lot HM 24928, were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden)

Phospholipid solutions

Egg-yolk phospholipids (70% phosphatidylcholine and 21% phosphatidylethanolamine) were prepared as described earlier in detail [3] The lipids were dispersed in a detergent-containing aqueous medium by vigorous stirring at pH 7–8 for 35 min at 22°C Dithioerythritol (DTE) was added, the pH was adjusted to 8 4 with NaOH and the mixtures were stirred overnight at 4°C Finally the mixtures were centrifuged for 2 h at 160000 × *g* at 2°C and floating material was discarded (cf Ref 1) The final concentrations were (phospholipid solution A100) 100 mM phospholipids, 125 mM cholate, 200 mM NaCl, 1 mM Na_2-EDTA, 2 mM DTE, 0 1 mM D-glucose and 20 mM Tris-HCl (pH 8 4) or (phospholipid solution B40) 40 mM phospholipids, 400 mM octyl glucoside, 200 mM NaCl, 1 mM Na_2-EDTA, 2 mM DTE and 20 mM Tris-HCl (pH 8 4)

Membrane protein solution

Human red cell membranes were prepared and stripped of peripheral proteins [4] The integral membrane proteins were solubilized at 8 mg/ml with 75 mM octyl glucoside in 2 mM DTE/50 mM Tris-HCl (pH 7 0) by stirring for 20 min at 2°C (cf Ref 3) Insoluble material was sedimented by centrifugation for 60 min at 160000 × *g* at 2°C The protein concentration in the supernatant was approx 4 mg/ml [3]

Methods

Dialysis-immobilization

Lipid vesicles were prepared and simultaneously entrapped in gel beads by dialysis of gel beads, either with cholate-solubilized lipids against 200 mM NaCl, 1 mM Na_2-EDTA, 1 mM 2-mercaptoethanol, 0 1 mM D-glucose and 20 mM Tris-HCl (pH 7 0) (buffer A) or with octyl glucoside-solubilized lipids against buffer A without glucose (buffer B)

The phospholipid solution A100 was diluted with buffer A either to 50 mM lipids and 62 mM cholate (phospholipid solution A50) or to 20 mM lipids and 25 mM cholate (phospholipid solution A20) Phospholipid solution B40 was diluted with buffer B to 20 mM lipids and 200 mM octyl glucoside (phospholipid solution B20) At these concentrations osmotic dilution was small during the dialysis described below (In some cases proteins were included) Then 3 0–3 5 ml of phospholipid solution A50, A20 or B20 was pumped into Sepharose 6B or Sephacryl S-1000 1 × 3 cm columns (2 5 ml) at a flow rate of 15 ml/h for Sepharose 6B and 20 ml/h for Sephacryl S-1000 The gels were then transferred directly from the columns into dialysis tubings, which were closed at one end and stretched out to flat shapes by the use of U-formed plastic frames (Fig 1) In this way we could easily pack the gel and the detergent was more rapidly removed from the mixture of gel beads and lipid solution, than in a cylindrical tubing The dialysis cells were covered with plastic lids and the bags were closed The samples were dialysed against 4 × 500 ml of buffer A or B with magnetic stirring for 2 5 days at 22°C After dialysis the gel beads were washed six times with dialysis buffer to remove all non-entrapped vesicles, as described in the legend to Fig. 2 The gels were then repacked into columns

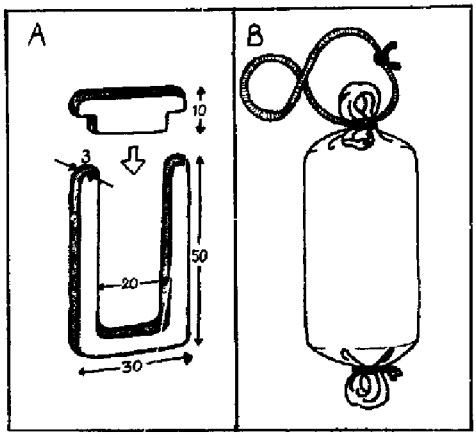

Fig 1 Dialysis cell (A) Perspex frame (distances in mm) used to give the dialysis membrane a flat shape (B) Dialysis cell A 15 cm-long dialysis tubing (circumference 60 mm), was slipped onto the close-fitting frame, the bottom end of the tubing was closed with a plastic string and the bag was filled with the dialysis mixture The frame was then covered with a lid and the top end of the tubing was closed

Characterization of entrapped vesicles

Vesicle amount The entrapped vesicles were eluted with 50 mM cholate and the phospholipid amount was determined (μmol of phospholipids per ml packed gel) by the ultramicromethod of Bartlett [5]

Vesicle volumes The total vesicle volume (internal volume together with lipid bilayer volume) was estimated by chromatography of D-[^{14}C]glucose on the column, first after entrapment of vesicles, and second, after solubilization of the vesicles and elution of the lipids with cholate (cf Ref 2) The internal volumes of

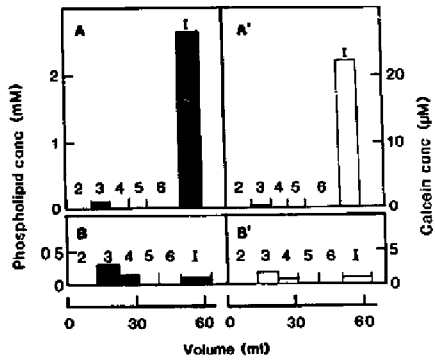

Fig 2 Dialysis-entrapment of phospholipid vesicles in Sepharose 6B (A, A') Vesicles formed and became entrapped in gel beads by dialysis of cholate-solubilized phospholipids (phospholipid solution A50, see Methods) and Sepharose 6B gel beads The dialysis mixture and buffer also contained 5 mM calcein After dialysis the gel beads were washed with dialysis buffer (without calcein) in six steps (1) the contents of the dialysis bag was transferred to a column in which the gel beads were packed, (2) the gel was washed in the column, (3)–(5) the gel was washed by centrifugations of the gel beads at 1000 × g for 3 × 10 min and (6) the gel beads were repacked and washed again in the column The washing volumes were collected and analyzed for phosphate and calcein. Bars 2–6 correspond to the washing volumes from steps (2)–(6) Filled bars show the phospholipid concentration and open bars show the calcein concentration. After washing, the entrapped vesicles were eluted with 50 mM cholate Bar I shows the eluate (B, B') To examine whether preformed vesicles could be trapped in or adsorbed to gel beads, vesicles were formed by dialysis of phospholipid solution A50 and then mixed with gel beads in a test-tube that was kept at 4°C for 24 h Oxidation of the vesicles by oxygen or light was avoided by storage of the gel beads in the dark under nitrogen gas The gel beads were washed and the vesicles were eluted as described above. Bars No 2–6 and bar 1 as above

the entrapped vesicles were determined after dialysis with 5 mM calcein in the dialysis mixture and buffer, followed by washings (cf Figs 2 and 3). Vesicle-entrapped calcein (cf. Refs 6 and 7) was released with 50 mM cholate and the concentration was determined fluorometrically using a standard curve (cf Ref 2)

Vesicle stability Vesicles were formed and entrapped in Sepharose 6B by dialysis of phospholipid solution A60 with gel beads The gel beads were washed as described above (and see legend to Fig 2) They were stored in a test-tube under nitrogen in the dark at 4°C for 9 days The gel was packed in a column After elution with dialysis buffer the released vesicles and phospholipids were determined by phosphorus analysis

Results

Dialysis-entrapment

Vesicles were formed and trapped in gel beads by dialysis of gel beads with detergent-solubilized lipids

After dialysis the gel beads were thoroughly washed in six steps to remove non-entrapped material completely (for details, see legend to Fig 2) Firstly, the gel beads were packed into a column and most of the non-entrapped material, corresponding to 80–95% of the original lipid amount, was removed The phospholipid and calcein concentrations in the washing volumes were determined for steps 2–6 (Figs 2 and 3) Typical results are shown for Sepharose 6B and Sephacryl S-1000 No phospholipids or calcein were removed in Step 2, i e , no entrapped vesicles were removed or broken in this chromatographic washing step (Figs 2 and 3, A and A') Next (steps 3–5), the gel beads were suspended in buffer and washed by centrifugation to remove vesicles or lipid aggregates that were trapped between or on top of the gel beads in the column The gel beads were then repacked into a column and washed without any loss of phospholipids or calcein (step 6) Most of these vesicles were removed in the first centrifugation (step 3) Finally, the entrapped vesicles were solubilized with cholate and the lipids were eluted with 50 mM cholate The bars I in Figs 2A and 3A thus represent the amount of phospholipids in the form of entrapped vesicles within gel bead pores (cf Tables I and II) These careful washing steps were used to prove that lipid vesicles had been entrapped in the gel pores In fact, to judge from the results of Fig 2A and 3A, only centrifugation procedures are needed to remove non-entrapped lipid vesicles

When preformed vesicles were mixed with gel beads only approx 1% of the vesicles, corresponding to 0 5 μmol of phospholipids per ml gel, were adsorbed to or in gel beads (Fig 2B) Dialysis of cholate-lipid solutions with Sephacryl S-1000 resulted in a small amount of

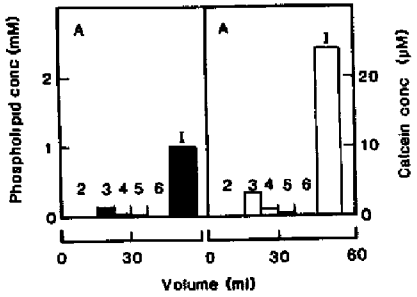

Fig 3 Dialysis-entrapment of phospholipid vesicles in Sephacryl S-1000 (A, A') Vesicles became trapped in Sephacryl S-1000 gel beads during formation by dialysis of octyl glucoside-solubilized lipids (phospholipid solution B20, see Methods) and gel beads The dialysis mixture and buffer contained 5 mM calcein The gel beads were washed after dialysis and the immobilized vesicles were eluted as described in the legend to Fig 2. Bars no 2–6 correspond to the washing volumes from washing step (2)–(6) and bar 1 shows the volume collected when the vesicles were eluted.

TABLE I

Entrapment of phospholipid vesicles and protein phospholipid vesicles in Sepharose 6B upon vesicle preparation by dialysis

Sepharose 6B gel beads were dialysed in the presence of given concentrations of lipids, cholate and red cell membrane proteins After dialysis the gel beads were washed as described in the legend to Fig 2 and then packed into a 2 5-ml column

Lipid conen (mM)	Protein conen (mg/ml)	Entrapped phospholipids (μmol/ml gel)	Internal vesicle volume (μl/μmol P)	Internal vesicle volume (μl/ml gel)	Vesicle diameter (nm)	Yield [c] (%)
20 [a]	–	2 8	3 6	10	120	15
20 [a]	0 8	2 0	n d [d]	n d	n d	12
50 [b]	–	9 5	1 6	15	60	19
50 [b]	2 0	6 5	n d	n d	n d	16

[a] 25 mM cholate (phospholipid solution A20)
[b] 62 mM cholate (phospholipid solution A50)
[c] Mol of entrapped phospholipids in percent of mol of detergent-solubilized lipids before dialysis
[d] Not determined

entrapped vesicles (0 1 or 2 2 μmol of phospholipids per ml gel, see Table II) The vesicles formed by dialysis of cholate-lipid mixtures [8] are small compared with the Sephacryl S-1000-pore size (approx 50–200 nm diameter) These results indicate that vesicles are mechanically trapped inside the pores

4% of the added [*carboxyl*-^{14}C]cholate remained in the dialysis bag after 2 5 days of dialysis of phospholipid solution A50, [*carboxyl*-^{14}C]cholate and Sepharose 6B gel beads The ensuing washings removed most of the residual cholate, 0 4% of the initial amount was found in the lipid fraction after elution with 50 mM cholate

Characterization of entrapped vesicle in Sepharose 6B

Phospholipid vesicles became entrapped in gel beads of Sepharose 6B to concentration of 2.8–9 5 μmol of phospholipids per ml gel upon dialysis of cholate-solubilized lipids in the presence of the gel beads The highest amount of entrapped vesicles was achieved when the dialysis mixture contained 50 mM lipids and 62 mM cholate (Table I) These vesicles had an internal volume of 1.6 μl per μmol of phospholipids, which corresponds to a vesicle diameter of 60 nm according to Fig 2 in Ref 9 The total volume was 1 7 μl/μmol of phospholipids A decrease in lipid concentration to 20 mM and cholate concentration to 25 mM, increased the size of the entrapped vesicles (Table I, cf Ref 8) Their average diameter was 120 nm and their internal volume was 3 6 μl per μmol of phospholipids However, the capacity for entrapment decreased to 2 8 μmol of phospholipids per ml gel. Obviously the effect of the increase in vesicle size was counteracted by the decrease in capacity, such that the total internal volume of entrapped vesicles per ml gel was larger in the first case (15 μl) than in the latter (10 μl) Protein-phospholipid vesicles became entrapped in amounts corresponding to 2 0 or 6 5 μmol of phospholipids per ml gel, that is, 70 ± 2% of the capacities found for phospholipid vesicles entrapped under similar conditions The yield of entrapped phospholipids was 12–19% and was always decreased by the presence of proteins in the dialysis

TABLE II

Entrapment of phospholipid vesicles and protein phospholipid vesicles in Sephacryl S 1000 upon vesicle preparation by dialysis

Sephacryl S-1000 gel beads were dialysed in the presence of given concentrations of lipids, detergent and protein. After dialysis the gel beads were washed as described in the legend to Fig 2 and then packed into a 2 5 ml column

Lipid conen (mM)	Protein conen (mg/ml)	Entrapped phospholipids (μmol/ml gel)	Internal vesicle volume (μl/μmol P)	Internal vesicle volume (μl/ml gel)	Vesicle diameter (nm)	Yield [d] (%)
20 [a]	–	3 0	7 3	22	230	18
20 [a]	0 8	3 5	n d	n d	n d	19
20 [b]	–	0 1	n d	n d	n d	0 9
50 [c]	–	2 2	1 0	n d	40	3 0

[a] 200 mM octyl glucoside (phospholipid solution B20)
[b] 25 mM cholate (phospholipid solution A20)
[c] 62 mM cholate (phospholipid solution A50)
[d] Moles of entrapped phospholipids in percent of moles of detergent-solubilized lipids before dialysis

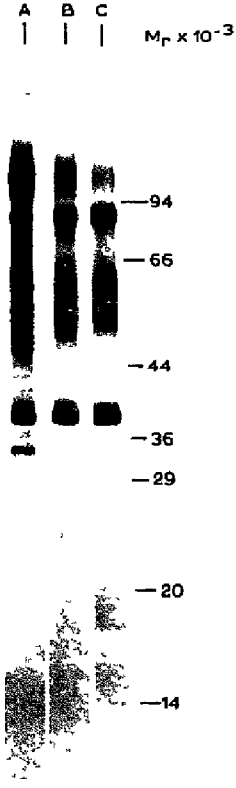

Fig 4 Sodium dodecyl sulfate electrophoresis in a gradient gel of polyacrylamide concentration 8–25% The electrophoresis was performed essentially as described in Ref 10 Sample volume 70 µl The gel was silver-stained (A) Purified membranes from human red cells, (B) integral membrane proteins from human red cells solubilized with octyl glucoside and added to the dialysis mixture, (C) proteins inserted in dialysis-immobilized vesicles in gel beads and eluted with cholate The main components are the anion transporter (apparent M_r 98000), the glycophorin A dimer (83000), the glucose transporter (53000–67000) and the glycophorin A monomer (38000)

mixture With proteins present the yield was 82 ± 2% of the yield achieved with phospholipids alone

Electrophoretic patterns of integral membrane proteins which probably became inserted in the lipid bilayers of the vesicles trapped upon dialysis of phospholipid solution A50, proteins and Sepharose 6B gel beads are shown in Fig 4, lane C Separate electrophoretic experiments (not illustrated) show that about 10% of the proteins were entrapped. The ratio between protein and lipid in the entrapped vesicles was therefore about 1 30 (w/w), which is consistent with protein-vesicle structures Lane B shows the patterns of the proteins which were included in the dialysis mixture Almost all proteins in the mixture were incorporated to the same extent An exception is the anion transport protein which seems to be present in smaller amount in the vesicles than in the original mixture and in the non-entrapped material (not shown) The large size of this protein may have reduced the yield in the reconstitution and entrapped procedure, since the concentration of anion transporter inside the gel beads is considerably lower than that outside Lane A shows the proteins present in the purified red cell membranes Some proteins are only partly solubilized under the conditions used in our experiments

During 9 days storage of Sepharose 6B with entrapped vesicles at 4 °C, 9% of the phospholipids were released

Characterization of vesicles entrapped in Sephacryl S-1000

The amount of entrapped phospholipid vesicles and protein-phospholipid vesicles in gel beads of Sephacryl S-1000 corresponded to 0 1–3 5 µmol of phospholipids per ml gel, as shown in Table II Dialysis of cholate-phospholipid solutions and gel beads resulted in entrapment of 0 1 or 2 2 µmol phospholipids per ml gel The yields of entrapped phospholipids were low, at most 3%

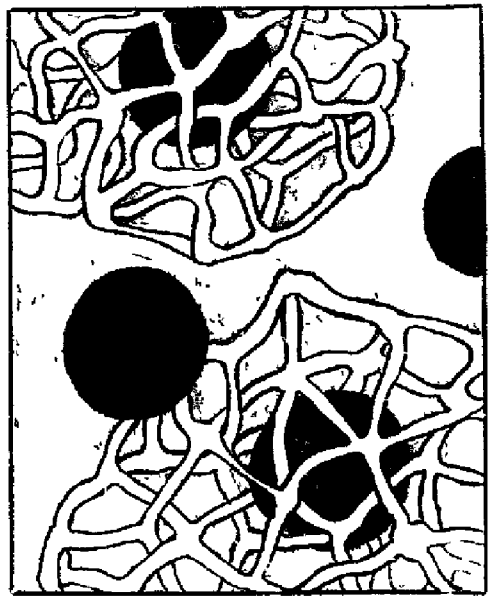

Fig 5 Schematic illustration of vesicles entrapped in pores of a gel bead matrix and of non-entrapped vesicles Only small parts of the gel beads are shown

Higher capacity, 3 0–3 5 μmol of phospholipids per ml gel, and a much higher yield, 18–19%, were achieved by dialysis of octyl glucoside-solubilized lipids and gel beads In this case, the entrapped vesicles had internal volumes of 7 3 μl per μmol phospholipids, which corresponds to diameters of approx 230 nm The total volume was estimated at 7 1 μl per μmol of phospholipid The capacity and the yield were increased when proteins were included in the octyl glucoside/lipid solution

Discussion

Entrapment procedure

The very thorough washings by both centrifugation and chromatography are probably not needed A simplified procedure is the following

(1) Phospholipid solution is pumped into a gel bead column A minimum amount of lipid solution will be needed and the dilution is minimal
(2) The lipid-gel bead mixture is transferred into a flat dialysis cell (Fig 1) and dialysed
(3) The gel beads with entrapped vesicles (Fig 5) are washed in three centrifugation steps to remove non-entrapped vesicles For chromatographic use the washed beads are packed into a column

Mechanism of entrapment

Vesicles became entrapped in gel beads upon dialysis of detergent-lipid solutions in the presence of gel beads The vesicles probably became trapped at the sites of their formation, upon becoming sufficiently large compared to the size of the pores (Fig 5) In general, the vesicle size can be controlled by choosing suitable lipid detergent ratios [8,11] The following results support the above interpretation (i) Preformed vesicles mixed with gel beads became immobilized, probably by adsorption to the gel matrix, in much lower amounts than were obtained upon dialysis-entrapment (ii) The amount of entrapped vesicles was dependent on the relative sizes of the vesicles and the gel bead pores The main limiting factor was that only vesicles present inside gel pores of suitable size became entrapped Vesicles formed outside the gel beads were, of course, not entrapped (iii) The vesicles that were trapped in the gel beads reduced the available volume inside the pores, and the properties of the gel as a medium for gel filtration were changed (unpublished results)

Applications

This method to entrap vesicles into gel beads provides a new type of chromatographic medium. Since no ligands or chemical treatments are used, new sites for non-specific binding should not be formed

Membrane proteins might easily be incorporated into the lipid bilayers of the entrapped vesicles for various studies by including them in the dialysis mixture Charged amphiphiles have been incorporated into entrapped vesicles in the same way and the charges have proved to be accessible for binding to proteins in ion-exchange chromatography experiments (unpublished results)

Eriksson and co-workers [12] have proposed that gel beads might be excellent carriers of vesicles in experiments where membrane proteins reconstituted into vesicles are studied and the protein-lipid vesicles should be separable from the incubation mixture Our method to entrap the vesicles in gel beads should be suitable and offers the advantage of reasonable vesicle stability

In the future, vesicles entrapped into suitable matrices may be used as carriers for drugs (or other agents) providing a slow release of, for instance, an antibiotic for treatment of burns

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